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Evaluation of Viral Infection in the Myocardium of Patients With Idiopathic Dilated Cardiomyopathy

Shigekazu Fujioka, MD, Yasushi Kitaura, MD, Akira Ukimura, MD, Hirofumi Deguchi, MD, Keishiro Kawamura, MD, Tadashi Isomura, MD, Hisayoshi Suma MD, Akira Shimizu, MD

Takatsuki and Kamakura, Japan

OBJECTIVES The aim of this study was to evaluate the viral etiology of idiopathic dilated cardiomyopathy (DCM).

BACKGROUND The demonstration of enteroviral genome in hearts with DCM has reinforced the importance of enteroviruses in the pathogenesis of DCM. However, there is uncertainty about the character and activity of enteroviruses detected in the myocardium. Recently, the association of hepatitis C virus or adenovirus with DCM has been reported.

METHODS Myocardial specimens from 26 patients with idiopathic DCM, which were obtained at partial left ventriculectomy (PLV), were examined virologically. Strand-specific detection of enteroviral RNA was performed to differentiate active viral replication from latent persistence. Polymerase chain reaction was used to detect genomic sequences of hepatitis C virus, adenovirus, cytomegalovirus, influenza viruses, mumps virus, herpes simplex viruses, varicella-zoster virus and Epstein-Barr virus.

RESULTS Plus-strand enteroviral RNA was detected in 9 (35%) of the 26 patients. Minus-strand enteroviral RNA was determined in seven (78%) of these nine plus-strand RNA-positive patients. Sequence analysis revealed that the enteroviruses detected were coxsackie B viruses, such as coxsackievirus B3 and B4. However, genetic material from other viruses was not detected. Six (86%) of seven minus-strand enteroviral RNA-positive patients died of cardiac insufficiency within the first six months after PLV.

CONCLUSIONS Coxsackie B viruses were seen in hearts with idiopathic DCM. Active viral RNA replication appeared to be present in a significant proportion of these cases. Minus-strand coxsackieviral RNA in the myocardium can be a marker for poor clinical outcome after PLV. There was no evidence of persistent infection by other viruses in hearts with DCM. (J Am Coll Cardiol 2000;36:1920–6) © 2000 by the American College of Cardiology

Enteroviruses have been implicated in the pathogenesis of idiopathic dilated cardiomyopathy (DCM) (1). This notion was supported originally by the long-term outcomes of some patients with acute myocarditis (2,3), retrospective serologic tests of viral antibodies (4–6) and studies of animal models (7–9). The demonstration of enteroviral RNA in hearts with DCM using slot blot (10), in situ hybridization (11) and polymerase chain reaction (PCR) (12,13) reinforced the importance of enteroviruses in the pathogenesis of DCM. However, more recent studies by PCR have yielded conflicting results. There was considerable variation in enteroviral positivity in hearts with DCM (14–22). In addition, there is uncertainty about the character of enteroviral genome detected in the myocardium. Considerable efforts have been made to clarify the nature of enteroviral genomes detected in hearts with DCM. Polymerase chain reaction with genotype-specific primers (16), stringent hybridization assay with genotype-specific probes (23), restriction enzyme digestion of PCR products (19), PCR single-strand conformation polymorphism (20) and nested PCR (N-PCR) followed by nucleotide sequence analysis (18,21,22) were used and provided limited information. Clarification of the genotypes of detected enteroviruses is the most important issue to be resolved in order to investigate the enteroviral etiology of DCM. Another point of interest is enteroviral activity in myocardium from patients with DCM. The differentiation between active enteroviral replication and latent persistence in the myocardium is considered to be important in view of the pathogenecity and management of this disease.

Links between cytomegalovirus or hepatitis C virus and DCM were reported (24,25). Recently, adenoviral DNA was detected in a significant proportion of pediatric patients with myocarditis (26) and in adult patients with DCM (27). However, subsequent studies, including molecular detection of these viruses in the myocardium, have not been performed. Because other viruses can also induce myocarditis (28), there is a possibility that other potentially cardiotropic viruses are associated with DCM. Therefore, it is necessary to examine whether other viruses or viral genomes can be demonstrated in the myocardium of patients with this disease.

Endomyocardial specimens have been primarily used for the detection of viral genomes (10–22,24–27). Because of the small size of myocardial samples, detailed virological studies, including the detection of a variety of viruses and

From the Third Division, Department of Internal Medicine, Osaka Medical College, Takatsuki, Japan; and Central Clinical Laboratory, Osaka Medical College, Takatsuki, Japan and Shonan Kamakura General Hospital, Heart Center, Kamakura, Japan.

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Abbreviations and Acronyms

cDNA	=	complementary DNA
DCM	=	dilated cardiomyopathy
N-PCR	=	nested polymerase chain reaction
NYHA	=	New York Heart Association
PCR	=	polymerase chain reaction
PLV	=	partial left ventriculectomy
RT-PCR	=	reverse transcription polymerase chain reaction

viral genomes, and the characterization of detected viruses have been difficult. In this study very large myocardial specimens obtained by partial left ventriculectomy (PLV) (29,30) were examined virologically to determine the viral etiology of idiopathic DCM. First, strand-specific detection of enteroviral RNA was carried out to differentiate between active enteroviral replication and latent persistence in DCM hearts. Sequence analysis of PCR products was also performed to characterize detected enteroviral genomes. Then we attempted to detect other potentially cardiotropic viruses including hepatitis C virus, adenovirus, cytomegalovirus, influenza viruses, mumps virus, herpes simplex viruses, varicella-zoster virus and Epstein-Barr virus by reverse transcription PCR (RT-PCR) or PCR. Another goal of this study was to determine the relationship between virological findings and the early prognosis of patients with idiopathic DCM after PLV.

METHODS

Patient population. Twenty-eight consecutive patients with idiopathic DCM who underwent PLV between April 1997 and October 1998 at the Shonan Kamakura General Hospital or Osaka Medical College Hospital were enrolled in this study. The clinical diagnosis of DCM was made according to the World Health Organization/International Society and Federation of Cardiology task force (31). Assessment of the symptoms of heart failure was performed using the New York Heart Association (NYHA) functional classification (32). All patients underwent noninvasive and invasive evaluation, including echocardiography (M mode, two-dimensional and Doppler) and cardiac catheterization with coronary angiography. Institutional review board approval was obtained at both hospitals, and all patients gave informed consent for inclusion in the study. All surgery was successfully carried out using the procedure described by Batista (29). All myocardial specimens resected during PLV were subjected to histopathological examination by light and electron microscopy and histochemistry. All samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until use for the detection of viral genomes. Left ventricular specimens from 21 normal individuals (15 men and 6 women; mean age, 49 ± 18 years) who died of noncardiac causes served as control specimens. They had normal histologic findings, and there was no evidence of cardiac viral infection. Left ventricular specimens from 15 patients (13 men and 2 women; mean age, 62 ± 15 years) who underwent aneurysmectomy or Dor's operation (33) were also studied.

Detection of viral genome. Total RNA and genomic/viral DNA were extracted from individual homogenates of myocardial specimens using ABI-340A Nucleic Acid Extractor (Applied Biosystems, Foster City, California) according to the standard program supplied by the manufacturer. Primer pairs to amplify the genomic sequences of the enterovirus (34) including coxsackie A and B viruses, echoviruses and polioviruses, hepatitis C virus (35), influenza A and B viruses (36), mumps virus (37), adenovirus (38), cytomegalovirus (39), herpes simplex 1 and 2 viruses, varicella-zoster virus (40) and Epstein-Barr virus (41) have been described previously. These primers were designed to amplify most virus types within their genus. Five μg of extracted total RNA was used for each viral first complementary DNA (cDNA) synthesis. Strand-specific viral RNA detection was performed for enterovirus (34) and hepatitis C virus (35). Conventional RT-PCR was used for the detection of genomic nucleic acid of mumps virus and influenza A and B viruses. For the detection of genomic nucleic acid of DNA viruses (adenovirus, cytomegalovirus, herpes simplex 1 and 2 viruses, varicella-zoster virus and Epstein-Barr virus), 10 μg of extracted DNA was used. Synthesis of viral first cDNA, PCR amplification of cDNA and Southern blot hybridization of the PCR products were performed as reported previously (34–41).

Independent RT-PCR and PCR were performed on each sample with beta-actin primers to evaluate a sufficient amount of extracted RNA and DNA (42). Several negative controls were included in each reaction. Extraction of total RNA and genomic/viral DNA, synthesis of viral first cDNA, PCR amplification of cDNA and Southern blot hybridization were performed in different rooms with separate equipment and pipettes.

Sequence analysis of the PCR product. Polymerase chain reaction amplified part of the 5′ noncoding region of the enteroviral genome (12), which is available for genotyping of enteroviruses (43). Polymerase chain reaction amplification products derived from DCM patients were visualized by agarose gel electrophoresis at ethidium bromide staining. Polymerase chain reaction products were gel-isolated and purified for sequencing using the Concert Gel Extraction Systems (Gibco-GRL, Rockville, Maryland). The nucleotide sequences were determined in each direction using an automated DNA sequencer with fluorescent dideoxy-chain terminators (PE-Applied Biosystems, Foster City, California) with the same primers that were used for PCR (12). The sequences were compared with all DNA sequences by FASTA search of GenBank database.

Virus isolation. Myocardial samples were homogenized in viral transport media, and the supernatant was used to inoculate susceptible cell lines for the isolation of viruses. The cell cultures were then incubated at 37°C and observed daily for 21 days for cytopathic effect.

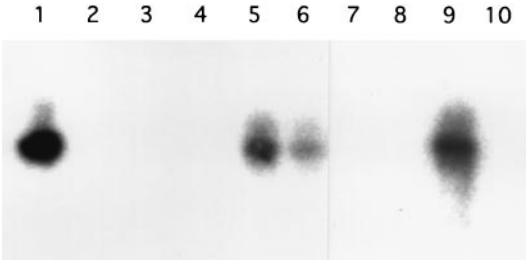

Figure 1. Representative Southern blot hybridization analysis of enteroviral polymerase chain reaction products. Strand-specific enteroviral RNA detection in the myocardium from four different patients is shown. In lanes 3, 5, 7 and 9, RT-PCR was performed with 3′ enteroviral primer for RT. The signals seen in lanes 5 and 9 correspond to enteroviral plus-strand cDNA. In lanes 4, 6, 8 and 10, RT-PCR was carried out with 5′ enteroviral primer for RT. The signal seen in lane 6 corresponds to enteroviral minus-strand cDNA. In summary, Patient 1 (lanes 5 and 6) shows active enteroviral replication (minus-strand detectable). Patient 9 (lanes 9 and 10) shows latent virus persistence (not minus-strand detectable). Patient 11 (lanes 3 and 4) and Patient 18 (lanes 7 and 8) are negative for enteroviral genome. Lane 1 = positive control of coxsackie B1 cloned DNA. Lane 2 = negative control. Patients No. 1, 9, 11 and 18; see Table 1. cDNA = complementary DNA; RT-PCR = reverse transcription polymerase chain reaction.

Viral genome and clinical outcomes. The results of viral genomes were compared with the clinical outcomes within one year after PLV. The clinical variables between virus genome-positive and virus genome-negative patients were also analyzed.

Statistical analysis. Statistica 5.1 statistical software was used for statistical analysis. Significance was determined by Fisher exact test or Student *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Clinical presentation. Two enrolled patients were found to have cardiac sarcoidosis and mitochondrial cardiomyopathy by histological examination of the resected myocardium and were excluded from further analysis. The remaining 26 patients (20 men and 6 women; mean age, 48 ± 15 years) were selected for this study. Seven patients were NYHA class III and 19 were NYHA class IV. All patients had enlarged left ventricular end-diastolic diameter and global left ventricular dysfunction assessed by echocardiography; mean left ventricular end-diastolic diameter was 80 ± 10 mm; mean left ventricular ejection fraction was 18 ± 7%. Angiography detailed no evidence of coronary disease. No patients had hypertension, drug or alcohol abuse, ischemic heart disease or systemic disorders. All specimens had a histological appearance consistent with a diagnosis of idiopathic DCM.

Detection of viral genome. First, strand-specific enteroviral RNA detection was performed to differentiate active viral replication and latent persistence. Figure 1 shows a representative Southern blot hybridization analysis of the amplified PCR products. Plus-strand enteroviral RNA was detected in 9 (35%) of the 26 patients. In seven (78%) of these nine plus-strand positive patients, minus-strand enteroviral RNA was demonstrated. However, genetic materials from hepatitis C virus, influenza viruses, mumps virus, adenovirus, cytomegalovirus, herpes simplex viruses, varicella-zoster virus and Epstein-Barr virus were not detected. No viral genomes were demonstrated in the control specimens. The PCR results for each virus were negative in all negative controls. All samples were assayed from the initial extraction of RNA and DNA at least three times and yielded the same PCR results. All samples were positive for the presence of beta-actin sequences by RT-PCR and PCR, indicating the successful extraction of both RNA and DNA.

Sequence analysis of enteroviral PCR product. Nucleotide sequence data were obtained on the 155 bp PCR products, a part of the 5′ noncoding region of the enteroviral genome. Figure 2 shows the sequence data from the patients with DCM. The amplified sequence from patient 1 was identical to that of coxsackievirus B3, strain Nancy. The sequence from patient 3 had the highest homology (99.6%) with that of coxsackievirus B4, strain J.V.B. In this case, one base substitution at position 546 (G to C) was recognized. In three cases (Patients 2, 6 and 7), the sequence exhibited significant homology (94.2%) with that of clinically isolated coxsackievirus B3 (44). Sequencing of the PCR products from the remaining five hearts was incomplete and resembled coxsackie B viruses.

Virus isolation. No viruses were isolated in cell culture by conventional methods in any of the hearts with DCM or control specimens.

Enteroviral genome and clinical outcome. Table 1 summarizes the enteroviral PCR results and clinical outcomes after PLV. Within one year after surgery, 17 of the 26 patients were alive, and most of them showed alleviation of their clinical symptoms. Six (67%) of the nine enterovirus-positive patients died of cardiac insufficiency within the first six months after PLV. These six patients had minus-strand enteroviral RNA in the myocardium. Therefore, six (86%) of the seven minus-strand enterovirus RNA-positive patients died of cardiac insufficiency within the first six months after PLV. However, 4 (24%) of the 17 enteroviral-negative patients and 4 (21%) of the 19 minus-strand RNA-negative patients died within a year after PLV. Mortality was statistically greater in enterovirus-positive patients than it was in enterovirus-negative patients ($p = 0.0425$). Minus-strand enterovirus RNA-positive patients had a particularly increased mortality rate compared with minus-strand RNA-negative patients ($p = 0.0053$). Clinical variables were compared between enterovirus RNA-positive and enterovirus RNA-negative patients. There were no significant differences in any clinical criteria between the two groups (Table 2).

DISCUSSION

Dilated cardiomyopathy is a myocardial disease characterized by ventricular dilation and impaired contractility with a poor prognosis. Partial left ventriculectomy was introduced

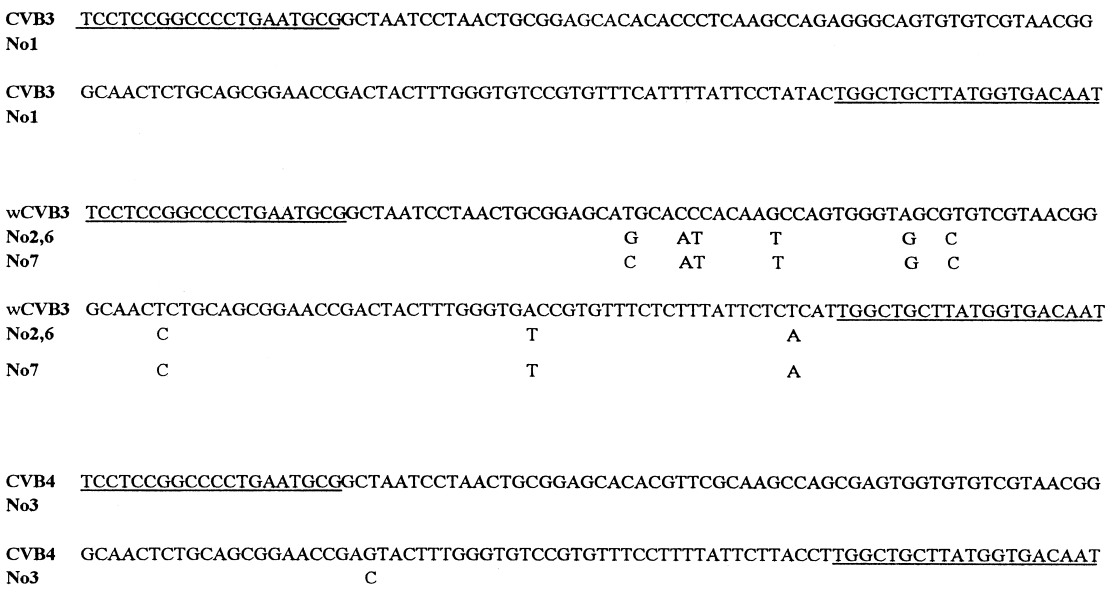

Figure 2. Alignment of nucleotide sequences of enterovirus polymerase chain reaction products derived from the myocardium of patients with idiopathic dilated cardiomyopathy (Patients No. 1, 2, 3, 6 and 7; see Table 1). Published sequences of coxsackievirus B3 (strain Nancy), coxsackievirus B3 (clinical isolate) and coxsackievirus B4 (strain J.V.B.) are shown in full, and any differences between these are indicated below by the presence of that base. A = adenine; C = cytocine; G = guanine; T = thymine; CVB3 = coxsackievirus B3 (strain Nancy); CVB4 = coxsackievirus B4 (strain J.V.B.); wCVB3 = coxsackievirus B3 (clinical isolate).

for the treatment of refractory DCM (29) and has received increasing worldwide attention (30). In this study using large myocardial samples obtained at PLV, detailed virological examination of hearts with idiopathic DCM was carried out to evaluate the viral etiology of DCM.

Detection and identification of enteroviral genome. First, enteroviral RNA was detected in 9 (35%) of the 26 patients with DCM. This incidence was relatively high compared with enteroviral positivity using endomyocardial biopsy samples in our laboratory (13,20) and others (12,14–

Table 1. Strand-specific Detection of Enteroviral Genome and Clinical Outcomes

Patient	Age/Gender	Enterovirus RNA		Outcomes
		Plus-strand	Minus-strand	
1	14 M	+	+	Died
2	53 M	+	+	Died
3	16 M	+	+	Died
4	65 M	+	+	Died
5	64 F	+	+	Died
6	36 M	+	+	Died
7	56 M	+	+	Alive
8	42 M	+	−	Alive
9	40 M	+	−	Alive
10	22 F	−	−	Died
11	61 M	−	−	Died
12	51 F	−	−	Died
13	49 M	−	−	Died
14	48 F	−	−	Alive
15	55 M	−	−	Alive
16	60 M	−	−	Alive
17	47 M	−	−	Alive
18	65 M	−	−	Alive
19	57 F	−	−	Alive
20	55 M	−	−	Alive
21	25 M	−	−	Alive
22	57 F	−	−	Alive
23	38 M	−	−	Alive
24	67 M	−	−	Alive
25	50 M	−	−	Alive
26	48 M	−	−	Alive

Table 2. Clinical Variables Between Patients With and Without Enterovirus RNA

	Enterovirus RNA Positive Patients (n = 9)	Enterovirus RNA Negative Patients (n = 17)	p Value
Age (yrs)	42.9 ± 18.8	50.3 ± 12.4	NS
Gender (male/female)	8/1	12/5	NS
NYHA functional class:			
III	2	5	NS
IV	7	12	
Echocardiographic data:			
LVDd (mm)	80.4 ± 7.9	79.9 ± 11.1	NS
LVEF (%)	17.8 ± 6.6	18.4 ± 7.4	NS
Surgical data:			
PLV			
With MVP/MVR	9	16	NS
With TAP	4	7	NS

LVDd = left ventricular end-diastolic diameter; LVEF = left ventricular ejection fraction; MVP = mitral valve plasty; MVR = mitral valve replacement; NYHA = New York Heart Association; PLV = partial left ventriculectomy; TAP = tricuspid valve plasty; NS = not significant.

19). Several factors exist that affect the frequency of enteroviral RNA such as differences in patient demography, stage of the disease and a difference in the detection method. However, this relatively high incidence of enteroviral RNA may be due to the size of the myocardial specimens examined. In situ hybridization studies showed uneven distribution of enteroviral RNA signals in the myocardium (11). In fact, an increased rate of enteroviral detection using multiple biopsy samples compared with those obtained with a single sample was reported (17). Therefore, the use of adequately sized samples is considered to be necessary to accurately determine the true frequency of enteroviral RNA in hearts with DCM. A significant rate of enteroviral RNA in the myocardium of patients with idiopathic DCM emphasizes the importance of enteroviruses as etiological agents of this disease.

Serological evidence indicates the presence of specific enteroviral serotypes (especially coxsackie B viruses) in hearts with DCM (4–6). However, there remains uncertainty concerning the types of enteroviral genomes detected in hearts with DCM. Several molecular biological techniques were used to differentiate detected enteroviral genomes (16,19,20,23). However, sequencing the enteroviral PCR products is most informative and can confirm the origin of the viruses. Three papers included sequence data on PCR products for patients with DCM (18,21,22). Two studies have drawn attention to the danger of contamination in N-PCR-based assay (18,21). Only Archard et al. (22) reported that the sequence of enteroviral N-PCR products had the highest homology with coxsackie B viruses. However, the study did not mention the specific types within the coxsackie B viruses. We demonstrated here, by nucleotide sequence analysis of one-stage PCR product, that viruses detected in hearts with DCM were coxsackie B viruses, such as coxsackievirus B3 and B4. Genomes of echovirus, coxsackie A virus and poliovirus were not detected, and the involvement of these viruses in hearts with DCM was deniable. Further investigation should, therefore, focus on coxsackie B viruses to elucidate the molecular basis of cardiotropism and pathogenesis.

Enteroviral activity in hearts with DCM. Transcription of minus-strand RNA from the plus-strand enteroviral genomic template is the essential first step of enteroviral replication. This minus-strand RNA is then used as a template to generate multiple copies of viral plus-strand genomes that are translated into enteroviral structural proteins and ultimately packaged into new virions. Therefore, the detection of minus-strand enteroviral RNA is an indicator of active enteroviral RNA replication (45). In this study minus-strand enteroviral RNA was verified in seven (78%) of nine plus-strand-positive patients. Therefore, our findings indicate that enteroviruses replicate actively in the myocardium in a significant proportion of cases of end-stage idiopathic DCM. This finding is extremely important in the investigation of the pathogenesis of and treatment for this disease. The persistence of enteroviral-specific Ig M response for many years in some patients with DCM was reported (6). Muir et al. (46) reported that enteroviral-specific IgM was detected in 22 (56%) of 39 patients with end-stage DCM. These data also support the idea that enterovirus persists in a replicative form in hearts with DCM.

Wessely et al. (47) reported that only a low level of expression of coxsackie B3 virus genome, not infectious virus progeny, was sufficient to induce a cytopathic effect in cultured neonatal rat ventricular myocytes. They also demonstrated that the transgenic expression of replication-restricted coxsackieviral genome in the heart could induce DCM (48). By analysis of isolated myocytes from these transgenic mice, defective-contraction coupling and a decrease in the magnitude of isolated cell shortening were observed. In this study enterovirus was not isolated in cell culture by conventional methods. It is possible that sequence mutations could accumulate during productive virus replication in the early phase of the disease and that mutant (defective virus) may appear in order to escape the host

immune systems. These mutants may persist in replicative form without formation of virus progeny, and restricted viral RNA replication in the myocardium could be capable of significantly impairing contractile function of the heart, as was shown in transgenic mice (48). Considering the high incidence of enteroviral positivity and active viral replication in end-stage hearts with DCM, the examination of enterovirus RNA in earlier stages of hearts with DCM would be recommended. If enteroviral RNA, especially minus-strand RNA, is demonstrated in the myocardium, an antiviral agent to coxsackie B virus (agent for preventing viral RNA replication) should be used for the management of this disease.

Other cardiotropic viruses and DCM. Genomes of hepatitis C virus, cytomegalovirus and adenovirus were detected in the myocardium of patients with DCM, and an association between these viruses and DCM was suggested (24,25,27). However, subsequent studies, including molecular detection of these viruses in the myocardium, have not been performed. In this study genomic materials of other potentially cardiotropic viruses, including hepatitis C virus, influenza viruses, mumps virus, adenovirus, cytomegalovirus, herpes simplex viruses, varicella-zoster virus and Epstein-Barr virus were not demonstrated. Therefore, persistent infection of these viruses in the myocardium of patients with DCM is unlikely. However, patients at earlier stages of the disease should be examined. The persistence of the immune response beyond clearance of the virus cannot be completely ruled out.

Clinical outcomes after PLV. It seems necessary to examine the prognosis in response to PLV. Enterovirus RNA-positive patients, especially minus-strand enteroviral RNA-positive patients, had an increased mortality rate compared with enterovirus RNA-negative patients. Several factors affecting outcome must be considered to determine whether enteroviral RNA, particularly minus-strand enteroviral RNA in the myocardium, is a predictor of outcome after PLV. There were no significant differences in preoperative clinical features, the severity of the heart failure, cardiac function or surgical results in enterovirus RNA-positive versus enterovirus RNA-negative patients. Therefore, our findings show that active coxsackieviral RNA replication in the myocardium can be a marker for poor prognosis after PLV.

Conclusions. Coxsackie B viruses were demonstrated in the myocardium of patients with idiopathic DCM. Active viral RNA replication in the myocardium appeared to be present in a significant proportion of these cases. Minus-strand coxsackieviral RNA in the myocardium can be a marker for poor clinical outcome after PLV. Considering these findings, an antiviral agent to coxsackie B virus should be used for the management of this disease. There was no evidence of persistent infection by other viruses, including hepatitis C virus, cytomegalovirus and adenovirus, in hearts with DCM.

Reprint requests and correspondence: Dr. Shigekazu Fujioka, Third Division, Department of Internal Medicine, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki, 569-8686 Japan.

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